GB1083815

Publication Title:

Vaccine adjuvants

Abstract:

Abstract of GB1083815

A vaccine for immunising a vertebrate against a protozoal disease comprises an antigen preparation of protozoal origin together with a saponin preparation. The vaccine may contain appropriate pharmaceutical excipients and may be sterile. The antigen preparation can be obtained from the causative protozoa, or from a morphological form other than that occurring in the vertebrate host. Antigen preparations for vaccines against disease caused by Trypanosoma spp. may be obtained from other species of protozoal flagellate non-pathogenic to the vertebrate species to be immunised. The antigen preparation can be obtained from protozoal cells grown in culture medium and, in many instances, from the surrounding medium. The cellular antigen preparation may be produced by killing the protozoal cells using conventional techniques. The medium itself may be used as an extra-cellular antigen preparation, or the antigenic material may be isolated therefrom. Data supplied from the esp@cenet database - Worldwide

---

1,083,815

PATENT SPECIFICATION

NO DRAWINGS

1,083,815

Inventor: RALPH ARTHUR NEAL.

Date of filing Complete Specification September 24, 1964.

Application Date October 1, 1963. No. 38695/63.

Complete Specification Published September 20, 1967.

Index at Acceptance: Class A5, B(4:8).
Int. Cl.: A 61 k 3/00

Vaccine adjuvants.

COMPLETE SPECIFICATION

We, THE WELLCOME FOUNDATION LIMITED, of 183-193 Euston Road, London, N.W.1, a company incorporated in England do hereby declare this invention for which we pray that a patent may be granted to us and the method by which it is to be performed, to be particularly described in and by the following statement:—

This invention primarily relates to the immunology of diseases of vertebrates caused by protozoa of the *Trypanosoma* spp. listed below (with the names of the causative protozoa): Chagas' disease (*Trypanosoma cruzi*), trypanosomiasis in man (*Trypanosoma gambiense* and *T. rhodesiense*), trypanosomiasis in ruminants (*Trypanosoma congolense, T. dimorphon, T. vivax, T. uniforme*), trypanosomiasis in pigs (*Trypanosoma simiae* and *T. suis*), nagana and surra in domestic mammals (*Trypanosoma brucei* and *T. evansi* respectively), and mal de caderas and dourine in equines (*Trypanosoma equinum* and *T. equiperdum* respectively), but may also be used in the prevention and treatment of other protozoal diseases, such as piroplasmosis (*Babesia* spp.), toxoplasmosis (*Toxoplasma gondii*) and blackhead of turkeys (*Histomonas maleagridis*).

Although a protozoal disease in a vertebrate is often associated with an immune response, attempts to use material from the causative protozoa to immunise by vaccination have not so far met with much success.

Protozoa are apparently only weakly antigenic, and although the antigenicity of weak antigens can often be enhanced by combining them with an aluminium compound or an oily emulsion, such classical vaccine adjuvants are relatively ineffective with protozoal antigens.

It has now been found that an effective vaccine for immunising a vertebrate against a protozoal disease can be produced if a saponin preparation is used as an adjuvant. According to the invention, there is provided a vaccine containing an antigen preparation of protozoal origin together with a saponin preparation. Other ingredients, including other adjuvants, may be present in the vaccine but the saponin preparation is essential. The invention further comprises an immunising set containing an antigen preparation of protozoal origin and a saponin preparation which may be packed separately and can be combined to produce a vaccine when required for use.

Saponins are complex glycosides which occur widely in plants. The individual substances are generally difficult to isolate and purify, however, and a given saponin preparation may thus be a mixture containing several chemically related types of saponin. The saponins (aglycones) are either triterpenoid or steroid derivatives, and the triterpenoid saponins are generally the more effective as protozoal vaccine adjuvants. Suitable saponin preparations may be obtained, for example, from the following plants:

Lemaireocereus thurberi (Cactaceae)
Viscum album (Loranthaceae)
Beta vulgaris (Chenopodiaceae)
Guajacum officinale (Zygophyllaceae)
Momordica cochinchinensis (Cucurbitaceae)
Aralia montana (Araliaceae)
Akebia quinata (Lardizabalaceae)
Clematis paniculata (Ranunculaceae)
Entada scandens (Papilionaceae)
Achras sapota (Sapotaceae)
Mimusops elengi (Sapotaceae)
Mora excelsa (Papilionaceae)
Styrax benzoin (Styracaceae)
Albizia gummifera (Minosaceae)
Aesculus bippocastanum (Hippocastanaceae)
Echinocystus fabacea (Cucurbitaceae)

Hedera helix (Araliaceae)
Sapindus saponaria (Sapindaceae)
Polyscias nodosa (Araliaceae)
Holboellia latifolia (Lardizabalaceae)
Quillaia saponaria (Rosaceae)
Saponaria officinalis (Caryophyllaceae)
Gypsophilia paniculata (Caryophyllaceae)
Agrostemma githago (Caryophyllaceae)
Glycyrrhiza glabra (Papilionaceae)
Hydrocotyle asiatica (Umbelliferae)
Cinchona spp. (Rubiaceae)

It is convenient to use a commercially available saponin preparation, for example from the bark of *Quillaia saponaria* or the root of *Saponaria officinalis*.

The antigen preparation can be obtained from the causative protozoa. Many pathogenic protozoal species have a series of morphological forms depending on the life cycle or the habitat, and an antigen preparation can often be obtained from a morphological form other than that occurring in the vertebrate host, culture forms are generally the most convenient.

Antigen praparations for vaccines against disease caused by *Trypanosoma* spp., particularly against Chagas' disease, may also be produced from other species of protozoal flagellate which are not pathogenic to the vertibrate species to be immunised, so that in some cases a safe non-pathogenic source of the antigen preparation is available.

The protozoa are grown by conventional means. Some must be harvested from infected animals but others can be grown in culture. The antigen preparation can be obtained from the protozoal cells and also in many instances from the surrounding medium, where there may be an extracellular antigen derived from the protozoa. A cellular antigen preparation can be produced by killing the cells under conditions compatible with the preservation of antigenic activity, for example successive freezing and thawing, freeze-drying, lysis by ultrasonic waves, or treatment with formaldehyde. The medium itself may be used as an extracellular antigen preparation, or the antigenic material may be isolated, concentrated, and purified by techniques applicable to biologically active substances of high molecular weight. It may be advantageous to include both cellular and extra-cellular components in the antigen preparation for the vaccine.

The products of the invention are produced by admixing the antigen preparation with a saponin preparation. A vaccine is conveniently produced by adding the saponin preparation in aqueous solution to a liquid or dry antigen preparation, and may be supplied liquid or freeze-dried. A convenient example of an immunising set according to the invention comprises a freeze-dried protozoal antigen preparation and a separate container of the saponin preparation, which can be combined together with any necessary liquid diluents to produce a vaccine when required.

The relative amounts needed of the antigen preparation and the saponin preparation depend on the immunological characteristics of the antigen, and the total amounts of each needed per dose depend also on the size of the vertebrate to be immunised and the nature of the disease. The amount of saponin used is below the level of systemic toxicity.

Immunisation is carried out by injecting the vaccine, the course of injections preferably comprising at least two doses spaced at least one week apart. One or more booster doses may eventually be required later for full immunity against challenge with the pathogenic protozoa.

The vaccines and components of immunising sets according to the invention are formulated for injection by the usual pharmaceutical methods. The formulations are sterile and may contain appropriate pharmaceutical excipients, for example bacteriostats and buffers and solutes to render the formulations physiologically compatible with body fluids.

The following examples illustrate the invention. The saponin preparation referred to as saponin SPL is a commercially available product obtained from *Quillaia saponaria* bark, and marketed by Messrs. Boake A. Roberts & Co. Ltd.

EXAMPLE 1

Antigen was prepared from trypanosomes separated from the blood of mice heavily infected with *Trypanosoma congolense* strain *NIMR*. An agglutinating agent (conveniently an antiserum against mouse red cells or a phytohaemagglutinin) was added to the blood to agglutinate the red cells. Many trypanosomes remained in the supernatant, and more were collected by washing the agglutinated red cells with cold glucose-saline. The trypanosomes were washed and resuspended in cold glucose-saline at $10^{8.3}$ trypanosomes/ml. The suspension was then rapidly frozen (using alcohol/solid carbon dioxide) and thawed three times to kill the trypanosomes, and the resultant antigen preparation was stored at −20°C until required for use. A liquid vaccine was prepared by mixing the antigen preparation with an equal volume of an aqueous solution of saponin SPL (1.0 mg./ml.).

The effectiveness of the vaccine was demonstrated in mice. Each mouse was given two subcutaneous immunising doses

of 0.2 ml. of vaccine two weeks apart, followed two weeks later by a subcutaneous challenge dose of $10^{5.4}$ living trypanosomes of *Trypanosoma congolense* strain *NIMR*. Untreated mice were given the same challenge and served as a control. Blood was taken from the tail of each mouse three times a week and examined microscopically for trypanosomes. In the untreated mice parasitaemia developed after a prepatent period of 3 to 5 days. In the immunised mice the onset of parasitaemia was delayed and some were still negative at 10 days. The results of five such experiments are shown below.

Treatment	Prepatent period (days) *Average*	*Maximum*	Mice negative *at 10 days*
Immunised	14	21	6/9
Control	5		0/10
Antigen without saponin	6		2/10
Immunised	14	21	5/9
Control	4		0/9
Immunised	13	21	4/8
Control	4		0/8
*Immunised	15	18	3/4
Control	5		0/5
°*Immunised	12	18	3/5
° Control	3		0/5

* Immunising injections four weeks apart
° Challenge dose $10^{6.0}$ trypanosomes
The maximum value indicated for the prepatent period means that the survival of mice beyond that time was not counted in calculating the average, which if approaching the maximum implies that many of the mice were alive at the end of the experiment.

EXAMPLE 2

A dry vaccine was produced by freeze-drying a liquid vaccine obtained as in Example 1. When required for use it was reconstituted in the volume of distilled water required to give a saponin concentration of 0.5 mg./ml.

The effectiveness of the vaccine was demonstrated as in Example 1 — average prepatent period 14 days (maximum 14, antigen without saponin 7), 10/10 mice negative at 10 days (antigen without saponin 2/10).

EXAMPLE 3

An antigen preparation obtained as in Example 1 was freeze-dried. A vaccine was produced by reconstituting the dry preparation in an aqueous solution containing saponin SPL (0.5 mg./ml.).

The effectiveness of the vaccine was demonstrated as in Example 1 — average prepatent period 14 days (maximum 14, antigen without saponin 7), 10/10 mice negative at 10 days (antigen without saponin 2/10).

EXAMPLE 4

An antigen preparation containing $10^{7.0}$ dead trypanosomes/ml. was obtained as in Example 1 and was mixed with an equal volume of an aqueous solution containing saponin SPL (1.0 mg./ml.) to produce a liquid vaccine.

The effectiveness of the vaccine was demonstrated as in Example 1. The results are shown below.

Treatment	Prepatent period (days) *Average*	*Maximum*	Mice negative *at 10 days*
Immunised	21	24	5/6
Control	7		1/7
Antigen without saponin	5		0/7

EXAMPLE 5

An antigen preparation obtained as in Example 1 was mixed with an equal volume of an aqueous solution containing potash alum (5.0 mg./ml.) and saponin SPL (1.0 mg./ml.) to produce a liquid vaccine.

The effectiveness of the vaccine was demonstrated as in Example 1 — average prepatent, period 9 days (maximum 21 control 4), 1/9 mice negative at 10 days (control zero).

EXAMPLE 6

An antigen preparation obtained as in

Example 1 was centrifuged at 9000g at 4°C into supernatant and sediment fractions. The sediment fraction was reconstituted in cold glucose-saline to contain the material from $10^{9.6}$ trypanosomes/ml. This antigen preparation was mixed with an equal volume of an aqueous solution containing saponin SPL (1.0 mg./ml.) to produce a liquid vaccine.

The effectiveness of the vaccine was demonstrated as in Example 1. The results are shown below.

Treatment	Prepatent period (days) *Average*	*Maximum*	Mice negative *at 10 days*
Immunised	21	21	8/8
Control	4		0/9
Antigen without saponin	6		1/9

EXAMPLE 7

A trypanosome suspension obtained as in Example 1 was treated with ultrasonic waves to kill the trypanosomes. The resultant antigen preparation was mixed with an equal volume of an aqueous solution containing potash alum (5.0 mg./ml.) and saponin SPL (1.0 mg./ml.) and freeze-dried to produce a dry vaccine.

The effectiveness of the vaccine was demonstrated as in Example 1 — average prepatent period 9 days (maximum 21, control 4), 2/8 mice negative at 10 days (control zero).

EXAMPLE 8

A trypanosome suspension obtained as in Example 1 was treated with formaldehyde at 1.0%. The resultant antigen preparation was mixed with an equal volume of an aqueous solution containing soponin SPL (1.0 mg./ml.) to produce a liquid vaccine.

The effectiveness of the vaccine was demonstrated as in Example 1 — average prepatent period 7 days (maximum 17, control 3), 1/5 mice negative at 10 days (control 0/8).

EXAMPLE 9

A vaccine was produced as in Example 8, but using formaldehyde at 0.1%.

The effectiveness of the vaccine was demonstrated as in Example 1 — average prepatent period 17 days (maximum 21, control 4), 7/9 mice negative at 10 days (control 0/10).

EXAMPLE 10

Trypanosomes were separated from infected blood as in Example 1. They were suspended at $10^{9.2}$ trypanosomes/ml. in glucose-saline and normal mouse serum and shaken at 37°C for 60 minutes. The suspension was centrifuged, and the antigen preparation obtained as the supernatant was mixed with an equal volume of an aqueous solution containing saponin SPL (1.0 mg./ml.) to produce a liquid cell-free vaccine.

The effectiveness of this vaccine was demonstrated as in Example 1 — average prepatent period 15 days (maximum 21, control 4), 4/9 mice negative at 10 days (control 0/9).

EXAMPLE 11

The whole blood of mice infected with *Trypanosoma congolense* strain *NIMR* was diluted with glucose-saline to contain $10^{9.0}$ trypanosomes/ml., and was rapidly frozen and thawed three times. The resultant antigen praparation was mixed with an equal volume of saponin SPL (1.0 mg./ml.) to produce a liquid vaccine.

The effectiveness of this vaccine was demonstrated as in Example 1 but using a challenge dose of $10^{6.0}$ trypanosomes — average prepatent period 16 days (maximum 18, control 3), 4/5 mice negative at 10 days (control 0/5).

EXAMPLE 12

The whole blood of mice infected with *Trypanosoma congolense* strain NIMR was treated with formaldehyde at 1.0%. The resultant antigen preparation was mixed with an aqueous solution of saponin SPL and freeze-dried to produce a dry vaccine which could be reconstituted to contain the material from $10^{8.0}$ trypanosomes/ml. and 0.5 mg. saponin/ml.

The effectiveness of this vaccine was demonstrated as in Example 1 — average prepatent period 19 days (maximum 21, control 4), 9/10 mice negative at 10 days (control 0/10).

EXAMPLE 13

Antigen was prepared from trypanosomes separated from the blood of mice infected with *Trypanosoma congolense* strain *Buswale*, using the procedure described in Example 1. The antigen preparation was mixed with an aqueous solution of saponin SPL and freeze-dried to produce a dry vaccine which could be reconstituted to contain the material from $10^{9.0}$ trypanosomes/ml. and 0.5 mg. saponin/ml.

The effectiveness of this vaccine was demonstrated in guinea pigs challenged with *Trypanosoma congolense* strain *F.N.* The infection in guinea pigs is less acute and more similar to the economically important bovine infection than it is in mice. The change in weight and the degree of infection were followed: 26 days after challenge the previously immunised guinea pigs (4) showed an average weight gain of

19 g. each while the controls (5) showed an average weight loss of 24 g. each.

EXAMPLE 14

A strain of *Trypanosoma congolense* freshly isolated from infected cattle (strain *K87*) was used to infect dogs. When the dog blood showed at least 10 trypanosomes per microscope field it was collected and heparin was added to prevent clotting. The red cells were agglutinated by adding 5 ml. of phytohaemagglutinin in solution to 100 ml. of blood. The mixture stood at room temperature for 15 minutes and was then spun at 1000 revolutions/minute for 5 minutes. The supernatant containing trypanosomes were removed. The agglutinated red cells were washed with glucose-saline until nearly all the trapped trypanosomes were recovered. The supernatants containing trypanosomes were pooled and spun at 2500 revolutions/minute for 15 minutes. The sediment, containing trypanosomes, white cells and some red cells was resuspended in glucose-saline at $10^{8.0}$ trypanosomes/ml. About 300ml. of infected blood provided about 60 ml. of this suspension (about 25% recovery of trypanosomes).

This suspension was rapidly frozen and thawed and saponin SPL was added as in Example 1 to produce a vaccine for immunising cattle. The vaccine was freeze-dried and then reconstituted before use.

EXAMPLE 15

Antigen was prepared from trypanosomes separated from the blood of mice infected with *Trypanosoma vivax* (rat-adapted strain), using the procedure described in Example 1. The antigen preparation was mixed with an equal volume of an aqueous solution of saponin SPL (1.0 mg./ml.) to produce a liquid vaccine.

The effectiveness of this vaccine was demonstrated in mice as in Example 1 but using a challenge dose of 10^6 trypanosomes of the same strain of *Trypanosoma vivax* — average prepatent period 17 days (maximum 21, control 3), 7/9 mice negative at 10 days (control 0/9).

EXAMPLE 16

An antigen preparation obtained as described in Example 15 was mixed with an equal volume of an aqueous solution containing potash alum (5.0 mg./ml.) and Saponin SPL (1.0 mg./ml.) to produce a liquid vaccine.

The effectiveness of this vaccine was demonstrated as in Example 15 — average prepatent period 21 days (maximum 21, control 3), 9/9 mice negative at 10 days (control 0/9).

In another experiment, the vaccine protected in six mice in a group challenged with the same strain of *Trypanosoma vivax* 10 weeks after immunisation.

EXAMPLE 17

Antigen was prepared from trypanosomes separated from the blood of mice infected with *Trypanosoma cruzi* strain *Y*, using the procedure described in Example 1. The parasitaemia in mice with *Trypanosoma cruzi* is much lower than with *Trypanosoma congolense*, however, and much less antigen was obtained. The antigen preparation containing $10^{8.8}$ dead trypanosomes/ml., was mixed with an equal volume of an aqueous solution of saponin SPL (1.0 mg./ml.) to produce a liquid vaccine.

The effectiveness of the vaccine was demonstrated in mice as in Example 1 using a challenge dose of $10^{5.3}$ trypanosomes of *Trypanosoma cruzi* strain *Y*. The results are shown below.

Treatment	Average *survival time*	Mice surviving *at 15 days*	Degree of *Infection*
Immunised	19 days	5/10	72
Control	12 days	0/10	186
Antigen without saponin	11 days	0/10	184

The figure for the average survival time has a maximum value of 33 days; survival of mice beyond that time was not counted in calculating the average which if approaching 33 days implies that many of the mice were alive at the end of the experiment. The degree of infection represents the number of trypanosomes seen in 20 high-power microscopical fields at the height of the parasitaemia. This applies also to the experiments of Examples 18 to 27.

EXAMPLE 18

An antigen preparation containing $10^{9.1}$ dead trypanosomes/ml. was obtained as in Example 17 and was mixed with an equal volume of an aqueous solution of saponin SPL (1.0 mg./ml.) to produce a liquid vaccine.

The effectiveness of the vaccine was demonstrated as in Example 17 using a challenge dose of $10^{5.0}$ trypanosomes — average survival time 33 days (control 16), degree of infection 16 (control 154).

In another experiment, similarly vaccinated mice surviving the challenge were completely resistant to another challenge eleven weeks later.

EXAMPLE 19

An antigen preparation containing $10^{9.4}$

dead trypanosomas/ml. was obtained as in Example 17 and was mixed with 0.25 volume of an aqueous solution of saponin SPL (1.0 mg./ml.) to produce a liquid vaccine.

The effectiveness of the vaccine was demonstrated as in Example 17 using immunising doses each of 0.5 ml. and a challenge dose of $10^{5.0}$ trypanosomes — average survival time 31 days (control 16) degree of infection 11 (control 154).

EXAMPLE 20

Antigen was prepared from trypanosomes separated from the blood of mice infected with *Trypanosoma cruzi* strain BG, using the procedure described in Example 1. The antigen preparation, containing $10^{8.0}$ dead trypanosomes/ml., was mixed with an equal volume of an aqueous solution of saponin SPL (1.0 mg./ml.) to produce a liquid vaccine.

The effectiveness of the vaccine was demonstrated as in Example 1 using a challenge dose of $10^{5.0}$ trypanosomes of *Trypanosoma cruzi* strain *BG* — average survival time 28 days (control 9), 8/10 survivors at 33 days (control 0/10 at 15 days), degree of infection 70 (control 306).

EXAMPLE 21

An antigen preparation containing $10^{9.4}$ dead trypanosomes/ml. was obtained as in Example 20 and was mixed with 0.5 volume of an aqueous solution of saponin SPL (1.0 mg./ml.) to produce a liquid vaccine.

The effectiveness of the vaccine was demonstrated as in Example 1 using immunising doses each of 0.3 ml. and a challenge dose of $10^{5.0}$ trypanosomes of *Trypanosoma cruzi* strain *Y* — average survival time 29 days (control 17), degree of infection 39 (control 251).

EXAMPLE 22

The blood of mice infected with *Trypanosoma cruzi* strain *BG* (containing $10^{7.7}$ trypanosomes/ml. plasma) was treated with an agglutinating agent for the red cells and the remaining liquid was centrifuged to remove trypanosomes and give an antigen preparation consisting of the cell-free plasma. This was mixed with 0.5 volume of an aqueous solution of saponin SPL (1.0 mg./ml.) to produce a cell-free liquid vaccine.

The effectiveness of the vaccine was demonstrated as in Example 1 using immunising doses each of 0.3 ml. and a challenge dose of $10^{5.0}$ trypanosomes of *Trypanosoma cruzi* strain *Y* — average survival time 32 days (control 13), degree of infection 42 (control 337).

EXAMPLE 23

Cell-free plasma was prepared from the blood of mice heavily infected with *Trypanosoma cruzi*, as in Example 22, and was fractionated by ammonium sulphate precipitation.

The plasma was first brought to 20%-saturation by adding 100%-saturated ammonium sulphate solution. It was centrifuged, and sediment was taken up in 4.0 ml. of distilled water and dialysed against demineralised water for 21 hours. It was again centrifuged and the sediment was taken up in 4.0 ml. of 1.0% aqueous sodium chloride (fraction "20PD" 3 mg. protein/ml.).

The 20%-saturated solution was brought to 40%-saturation by adding 100%-saturated ammonium sulphate solution. It was centrifuged, and the sediment was taken up in 4.0 ml. of distilled water and dialysed for 21 hours against dimineralised water and then again centrifuged. The supernatant was decanted (fraction "40SD", 16 mg. protein/ml.). The sediment was taken up in 4.0 ml. of 1.0% aqueous sodium chloride (fraction "40PD", 15.5 mg. protein/ml.).

The 40%-saturated solution was brought to 60%-saturation by adding 100%-saturated ammonium sulphate solution. It was centrifuged, and the sediment was taken up in 4.0 ml. of 1.0% aqueous sodium chloride and dialysed for 21 hours against demineralised water. The precipitated protein was redissolved by adding 40 mg. of sodium chloride (fraction "60SD", 65 mg. protein/ml.).

The 60%-saturated solution was brought to 80%-saturation by adding solid ammonium sulphate. It was centrifuged, and the sediment was taken up in 4.0 ml. distilled water and dialysed for 21 hours against dimineralised water. The precipitated protein was redissolved by adding 40 mg. of sodium chloride (fraction "80SD", 40 mg. protein/ml.).

The 80%-saturated solution was brought to 100%-saturation by adding solid ammonium sulphate. It was centrifuged, and the sediment was taken up in 4.0 ml. distilled water and dialysed for 21 hours against dimineralised water. The precipitated protein was redissolved by adding 10 mg. of sodium chloride (fraction 100SD, 2 mg. protein/ml.).

The 100%-saturated solution was also dialysed for 21 hours against demineralised water and tested for antigenicity.

The fractions were mixed with saponin SPL to form liquid vaccines. The vaccines from several fractions were effective, although fraction 40PD gave the best suppression of trypanosomes. This was demonstrated as in Example 1 with immunising doses each of 0.3 ml. (containing the equivalent of 0.2 ml. of plasma and 0.1 mg. saponin) and a challenge dose of the trypanosomes of the same strain of

Trypanosoma cruzi — average survival time 33 days (control 20 days), degree of infection 29 (control 272). Two of the eight vaccinated mice remained free from infection.

EXAMPLE 24

HeLa cells containing *Trypanosoma cruzi* strain *Y* in the leishmania stage were separated from the glass on which they grew and from each other by treatment with 0.02% ethylenediaminetetracetate buffer solution. This was washed off with buffered glucose-saline. The cells were suspended in a known volume of glucose-saline and counted. Smears of the suspension were then stained so that the number of parasites/cell could be counted. The volume was adjusted to contain $10^{8.0}$ leishmania/ml. The suspension was then rapidly frozen and thawed three times to kill the cells and parasites. The resultant antigen preparation was mixed with an equal volume of an aqueous solution of saponin SPL (1.0 mg./ml.) to produce a liquid vaccine.

This vaccine was effective in protecting mice against a challenge dose of the trypanosomes of *Trypanosoma cruzi* strain *Y*.

EXAMPLE 25

Trypanosoma cruzi strain *Y* was grown in the crithidial form in glucose-saline overlying nutrient agar which contained 20% defibrinated horse blood. A suspension of the washed crithidia at $10^{7.5}$/ml. in glucose-saline was rapidly frozen and thawed three times. The resultant antigen preparation was mixed with an equal volume of an aqueous solution of saponin SPL (1.0 mg./ml.) to produce a liquid vaccine.

The effectiveness of the vaccine was demonstrated as in Example 1 using a challenge dose of $10^{5.0}$ trypanosomes of *Trypanosoma cruzi* strain *Y* — average survival time 23 days (control 12), degree of infection 187 (control 852).

EXAMPLE 26

Antigen was prepared from the crithidia of *Trypanosoma melophagium* using the procedure described in Example 25. The antigen preparation, containing $10^{8.0}$ dead crithidia/ml., was mixed with an equal volume of an aqueous solution of saponin SPL (1.0 mg./ml.) to produce a liquid vaccine.

The effectiveness of the vaccine against *Trypanosoma cruzi* infections was demonstrated as in Example 1 using a challenge dose of $10^{4.0}$ trypanosomes — average survival time 24 days (control 15), 5/10 mice surviving at 33 days (control 0/10), degree of infection 101 (control 128).

EXAMPLE 27

Trypanosoma melophagium was grown by the procedure described in Example 25 to a concentration of $10^{8.5}$ crithidia/ml. The crithidia were spun down to leave an antigen preparation consisting of the liquid nutrient medium in which the crithidia had grown. This was mixed with 0.1 volume of an aqueous solution of saponin SPL to produce a cell-free liquid vaccine.

The effectiveness of the vaccine against *Trypanosoma cruzi* infections was demonstrated as in Example 1 using a challenge dose of 10^5 trypanosomes of *Trypanosoma cruzi* strain *Y* — average survival time 27 days (control 17), degree of infection 170 (control 259).

EXAMPLE 28

A liquid vaccine was produced from the leptomonads of *Leptomonas collosoma* using the procedure described in Example 25.

This vaccine resembled the product of Example 25 in its effectiveness against *Trypanosoma cruzi* infections in mice.

EXAMPLE 29

A liquid vaccine was produced from the nutrient medium in which the leptomonads of *Leptomonas collosoma* had grown, using the procedure of Example 27.

This vaccine was effective against *Trypanosoma cruzi* infections in mice.

EXAMPLE 30

A liquid vaccine was produced from the crithidia of *Crithidia fasiculata* using the procedure described in Example 25.

This vaccine resembled the product of Example 25 in its effectiveness against *Trypanosoma cruzi* infections in mice.

EXAMPLE 31

A liquid vaccine was produced from the nutrient medium in which the crithidia of *Crithidia fasciculata* has grown, using the procedure of Example 27.

This vaccine was effective against *Trypanosoma cruzi* infections in mice.

EXAMPLE 32

A liquid vaccine was produced from the crithidia of *Trypanosoma mega* using the procedure described in Example 25.

This vaccine was effective against *Trypanosoma cruzi* infections in mice.

EXAMPLE 33

Antigen was prepared from trypanosomes separated from the blood of mice infected with *Trypanosoma evansi*, using the procedure described in Example 1. The antigen preparation was mixed with an equal volume of an aqueous solution of saponin SPL (1.0 mg./ml.) to produce a liquid vaccine.

This vaccine protected mice against subsequent challenge with the trypanosomes of *Trypanosoma evansi*.

WHAT WE CLAIM IS:—

1. A vaccine comprising a non-patho-

genic antigen preparation of protozoal origin together with a saponin preparation.

2. An immunising set comprising preparations containing a non-pathogenic antigen of protozoal origin and a saponin, which may be packed separately, to be used in a combined form as a vaccine containing both.

3. A vaccine or an immunising set according to either of claims 1 and 2, in which the antigen preparation is obtained from protozoa of the *Trypanosoma species.*

4. A vaccine or an immunising set according to claim 3, in which the antigen preparation is obtained from protozoa of *Trypanosoma cruzi.*

5. A vaccine or an immunising set according to claim 3, in which the antigen preparation is obtained from protozoa of *Trypanosoma congolense.*

6. A vaccine or an immunising set according to claim 3, in which the antigen preparation is obtained from protozoa of *Trypanosoma evansi.*

7. A vaccine or an immunising set according to any of the preceding claims in which the saponin is obtained from the bark of *Quillaia saponaria* or the root of *Saponaris officinalis.*

8. A vaccine or an immunising set according to any of the preceding claims, in which the antigen preparation is obtained from protozoa killed by a treatment with formaldehyde or with ultrasonic waves.

9. A vaccine or an immunising set according to any of claims 1 to 7, in which the antigen preparation is obtained from protozoa killed by freeze-drying or successive freezing and thawing.

10. A vaccine or an immunising set according to any of the preceding claims, in which any of the preparations is presented in a liquid vehicle.

11. A vaccine or immunising set according to any of the claims 1 to 9, in which the antigen preparation is in a freeze-dried form.

12. A method for the production of a vaccine, which comprises the admixture of a non-pathogenic antigen preparation of protozoal origin with a saponin preparation.

13. A method according to claim 12, in which the antigen preparation is obtain from protozoa of the *Trypanosoma* species.

14. A method according to claim 13, in which the antigen preparation is obtained from protozoa of *Trypanosoma cruzi.*

15. A method according to claims 13 in which the antigen preparation is obtained from protozoa of *Trypanosoma congolense.*

16. A method according to claim 13, in which the antigen preparation is obtained from *Trypanosoma evansi.*

17. A method according to any of the claims 12 to 16, in which the saponin is obtained from the bark of *Quillaia saponaria* or the root of *Saponaria officinalis.*

18. A method according to any of the claims 12 to 16, in which the antigenic preparation is obtained from protozoa killed by a treatment with formaldehyde or with ultrasonic waves.

19. A method according to any of claims 12 to 16, in which the antigenic preparation is obtained from protozoa killed by freeze-drying or successive freezing and thawing.

20. A method according to any of claims 12 to 19, in which any of the preparations is in a liquid vehicle.

21. A method according to any of claims 12 to 19, in which the antigen preparation is in a freeze dried form.

22. A method for the production of a vaccine substantially as described with reference to Examples 1 to 35.

23. A method for the prevention and treatment of a protozoal disease in vertibrate animals excluding human beings, which comprises an immunisation with a vaccine containing a non-pathogenic antigen preparation of protozoal origin together with a saponin preparation.

R. F. HASLAM,
Agent for the Applicants.
Chartered Patent Agent.

Sheerness: Printed for Her Majesty's Stationery Office, by Smiths Printers and Duplicators.—1967.
Published at the Patent Office, 25 Southampton Buildings, London, W.C.2, from which copies may be obtained.